Madroño, Vol. 56, No. 4, pp. 221–228, 2009

IN VITRO PROPAGATION, CRYOPRESERVATION, AND GENETIC ANALYSIS OF THE ENDANGERED *HEDEOMA TODSENII* (LAMIACEAE)

Valerie C. Pence, G. Douglas Winget, Kristine M. Lindsey, Bernadette L. Plair, and Susan M. Charls
Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo & Botanical Garden, 3400 Vine St., Cincinnati, OH 45220
valerie.pence@cincinnatizoo.org

Abstract

Todsen's pennyroyal (*Hedeoma todsenii* R. S. Irving, Lamiaceae) is a federally endangered species from the mountains of south central New Mexico that rarely produces seed. In vitro propagation methods were developed to provide material for cryostorage and for reintroduction, if that becomes necessary. Cultures were initiated from shoot tips taken from the ex situ collection at The Arboretum at Flagstaff, resulting in 12 genetic lines that were maintained on MS medium with 0.1 mg/L BAP and 0.01 mg/L NAA. Tests with other media commonly in use in CREW's Endangered Plant Propagation Program indicated that MS medium with 0.5 mg/L BAP increased shoot production and MS medium with 0.5 mg/L IBA increased root production over the maintenance medium. Other concentrations of IBA tested did not improve rooting, and a pulse of IBA followed by culture on charcoal-containing medium did not increase rooting significantly above the control. Approximately half of the plants moved to soil survived acclimatization, regardless of previous treatments. Survival through cryopreservation averaged 35% with no significant difference between the encapsulation dehydration and encapsulation vitrification procedures, and shoot tips from all 12 lines have been banked in liquid nitrogen for long-term storage. RAPD analyses indicated that there was less diversity among plants that exist in close proximity in situ than among genotypes that are separated by more distance. These propagation, cryopreservation, and genetic analysis protocols are all methods that can be used as tools to provide support for the long-term conservation of this species.

Key Words: Cryopreservation, endangered plant, *Hedeoma todsenii*, in vitro, RAPD.

Hedeoma todsenii R.S. Irving (Todsen's pennyroyal, Lamiaceae) (Irving 1979) is a federally endangered species found in three population clusters in the Sacramento and San Andres Mountains of south central New Mexico. Because of its remote locations, most of the threats to this species are natural, including fire, climate change, limited natural habitat, and limited sexual reproduction. However, changes in land management, illegal grazing, and military activities may also pose threats (U.S. Fish and Wildlife Service 2001).

Seeds of this species are rarely available for propagation or preservation. *H. todsenii* has been shown to be capable of out-crossing, but it appears to have a low percentage of flowers producing seed, a very low number of seeds per flower, and a low germination rate in the laboratory (Huenneke 1993). This study was undertaken to develop in vitro propagation and cryopreservation methods for *H. todsenii* to supplement traditional ex situ conservation methods and in situ conservation efforts.

In vitro methods can provide a variety of tools for conservation. These include in vitro collecting (IVC), a method for initiating tissue cultures in the field (Pence et al. 2002). IVC can be used when seeds are not available and when conditions for the transport of cuttings may not be optimal. In vitro propagation methods can then be used to increase the number of plants available for reintroduction, research, education, etc. When seeds are not available for germplasm storage, shoot tips from in vitro cultures can be cryopreserved, and this can be an alternative method for long-term germplasm conservation.

The growth of *H. todsenii*, as large mats, and its low seed production and germination rate have led to speculation that the plants are primarily reproducing asexually in the wild. Comparing DNA sequence information could show whether they do in fact reproduce clonally (Sydes and Peakall 1998). Random amplified polymorphic DNA (RAPD) has been used to reveal DNA sequence differences between species and even those more subtle differences associated with hybrids, ecotypes, or individual members of a population (Krasnyanski et al. 1998; Liu et al. 2006). Analysis of the number of identical DNA bands between individuals can be used to group plants (Adams et al. 2003). Comparison of these groupings to the geographical locations of the plants can determine the apparent genetic diversity, gene flow and genetic interaction within and between separated populations (Morden and Loeffler 1999; Skoula et al. 1999; Khanuja et al. 2000). This information can prove invaluable in cases where an endangered species is being

prepared for reintroduction (Maki and Horie 1999; Mattner et al. 2002). In the case of *H. todsenii*, which appears to rely heavily on vegetative reproduction, it would be of interest to know the extent and distribution of genetic variation within the species.

This study describes the application of in vitro methods to the propagation and preservation of *H. todsenii*. In addition, it describes the results of RAPD studies on the in vitro lines established and the genetic relationships of the populations from which they originated.

Methods

Establishment of Cultures

Shoots of *Hedeoma todsenii* were obtained from the ex situ collection at The Arboretum at Flagstaff, and cultures were initiated twice using two different methods.

Method 1. Multiple shoots were collected from each of four plants and sent overnight to the Center for Conservation and Research of Endangered Wildlife (CREW). These were then surface sterilized with a 1:20 dilution of commercial bleach with 0.05% Tween 20 for 10 min, followed by 2 rinses in sterile, pure water. The shoots were then placed onto medium in 25 × 150 mm borosilicate culture tubes with colorless polypropylene closures, 15 ml of medium/tube. The medium consisted of Murashige and Skoog (1962) salts and minimal organics (Linsmaier and Skoog 1965) (PhytoTechnology Laboratories, Shawnee Mission, KS), 3% sucrose, 0.22% gelrite (Kelco, San Diego, CA) (=MS basal medium), with 0.5 mg/L benzylaminopurine (BAP), 0.05 mg/L naphthaleneacetic acid (NAA), and 100 mg/L active benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, 95%) (Sigma-Aldrich, St. Louis, MO). This medium was similar, but not identical, to the medium reported as effective in propagating a related species, *H. multiflorum* (Koroch et al. 1997). Multiple shoots were cultured from each of the four genetic lines received. Contamination was monitored visually, and when it was found in some of the original isolates, the tissues were cut to remove contaminated areas, re-sterilized and placed on the same medium with the addition of 500 mg/L carbenicillin and 200 mg/L cefotaxime; both were filter sterilized and added to the autoclaved medium (all antibiotics were from Sigma-Aldrich). Even though there was contamination in some of the cultures, some material from each genetic line survived to establish lines HT-1 to HT-4.

Method 2. This method used the technique of IVC (in vitro collecting) (Pence 2005). One shoot tip was collected from each of 55 documented genotypes. These were surface sterilized in the field by wiping with 70% ethanol and were placed into 7 ml borosilicate scintillation vials. Each vial contained 2.5 ml of the same medium used previously, except that antibiotics were added as a drop (0.05 ml) of a freshly made, filter sterilized solution (of 0.25 mg/ml vancomycin and 5 mg/ml cefotaxime) into each vial, after the tissue was added. The vials were then sent overnight to CREW where they were evaluated for browning and contamination. The 36 shoots that remained clean and green after a week were transferred to fresh medium of the same formulation, but without fungicide or antibiotics. Nine (25%) of these shoots initiated growth, and these were subcultured onto GM medium (Table 1) and maintained as lines HT-5 to HT-13. One line was lost in subsequent culture, and lines HT-1 to HT-12 were used for the growth experiments described here. The survival of only 9 genotypes from the original 55 collected resulted from the fact that only one sample of each genotype was collected. Other work in this laboratory has demonstrated that collecting multiple samples of a particular genotype generally ensures survival of at least one sample from which cultures can be established (Pence 2005). At the time of the *H. todsenii* collections, the development of propagation protocols was the goal, rather than the initiation of multiple genotypes. In subsequent collections, multiple samples have been cultured from each genotype, when possible.

Shoot Propagation and Rooting

Propagation and further experiments with these cultures centered on the use of three media that have been in standard use in the Endangered Plant Propagation Program at CREW. All were MS basal medium with 0.8% agar replacing gelrite, with no antibiotics or fungicide. The media included a low hormone, general maintenance medium (GM medium); a second propagation medium with a higher level of cytokinin (P medium); and a rooting medium (R medium) (Table 1). These were tested with *H. todsenii* to quantify their effects on growth and rooting. Stock maintained on GM medium was used for all experiments. Cultures in tubes, as described above, were maintained at 26°C, under Cool-White fluorescent lights, 16:8 hrs light:dark cycle, at approximately 40–60 µmol/m2/s photosynthetically active radiation (PAR). All genotypes were labeled and maintained separately.

In Experiment 1, shoot and root production were compared on GM, P, and R media. Shoots of all 12 lines were cultured on all three media, 20 shoots of each line on GM and R media and 10 shoots of each line on P medium. The number of shoots produced per culture, the number of shoots with roots, and the number of roots per shoot were recorded after two months. This

TABLE 1. GROWTH REGULATOR CONCENTRATIONS IN MEDIA FOR PROPAGATING *H. TODSENI* TESTED IN TWO EXPERIMENTS. 1) A comparison of three media commonly used in this laboratory; and 2) a comparison of four concentrations of IBA. All media contained MS basal medium with 0.8% agar.

Test	Medium	Growth regulator (mg/L)		
		BAP	NAA	IBA
Experiment 1	GM	0.1	0.01	—
	P	0.5	—	—
	R	—	—	0.5
Experiment 2	0 IBA	—	—	0
	0.5 IBA	—	—	0.5
	2 IBA	—	—	2
	10 IBA	—	—	10

experiment was done twice with all 12 genetic lines. The shoot multiplication rate for stem pieces with nodes vs. shoot tips was also determined using GM medium alone, and the number of shoots resulting after two months of culture was recorded. This experiment was also done with all 12 genetic lines.

In Experiment 2, media with MS basal medium with agar and either 0, 0.5, 2 or 10 mg/L IBA were tested. Although this was done specifically to enhance rooting, the shoot multiplication rate, the number of shoots with roots, and the number of roots per shoot were all recorded after two months. This experiment was done twice with all 12 genetic lines, 10 shoots of each line on each of the four media per replicate. An alternative method for rooting was also tested, using a 7-day pulse on MS basal medium plus agar and 0 or 50 mg/L IBA followed by culture on Woody Plant (WP) basal medium (Lloyd and McCown 1980) plus 0.8% agar and 0.05% activated charcoal (Sigma-Aldrich). After two months on charcoal, the presence or absence of roots was scored, and this experiment was done twice with each of the 12 genetic lines of *H. todsenii*.

Acclimatization

Rooted shoots were carefully removed from culture, rinsed to remove medium from the roots, and planted in a moist 4:1:1 mix of commercial play sand: soil mix (a coarse, custom soil mix, Ammon Wholesale Nursery, Burlington, KY): clay (from a wooded area in southwestern Ohio). Because of its availability, an autoclave was used to sterilize the soil. Soil was approximately 3 cm deep, in 10 × 10 × 11.5 cm polystyrene culture boxes (Phytotech) with lids. These were incubated in the laboratory under fluorescent lights (a mix of Gro-Lux and CoolWhite), 16:8 hr light:dark cycle, 45 ± 10 μmol/m²/s PAR, at 21–23°C and ambient humidity outside the boxes. Each container had four, 1 cm diam. drainage holes in the bottom and one, 2 cm diam. Sun Biofilter membrane (Sigma-Aldrich) over a hole approximately 1 cm in diam. in the lid. The plants were monitored carefully and the boxes watered with purified (reverse osmosis) water when needed to avoid the chlorine and calcium in the local water source. The plants were carefully watched, and if they remained green and unwilted, after 5 wk the lids were loosened and opened slightly. Lids were opened more every 2–3 d until the plants appeared stable and acclimatized, at which time the lids were removed completely. Survival of plants taken from an IBA pulse experiment and from the IBA concentration experiment was compared after 7 wk.

Cryopreservation of Shoot Tips

Shoot tips, approximately 1 mm in length, were isolated for cryopreservation from cultures 34 to 51 d in age that had been grown on MS basal medium with 0.5 or 1.0 mg/L BAP. After isolation, shoot tips were placed onto GM medium plus 0.3 M mannitol and 10 μM abscisic acid (ABA) with 0.8% agar in 60 × 15 disposable petri plates, 15 ml of medium per plate, for a preculture of 48 hr. Shoot tips were then prepared for liquid nitrogen (LN) exposure using either the encapsulation dehydration procedure (10 trials) (Fabre and Dereuddre 1990) or the encapsulation vitrification procedure (24 trials) (Hirai et al. 1998). With the encapsulation dehydration procedure, 5–10 tips each were recovered as controls after the preculture, encapsulation, and pretreatment steps, while 10–20 tips each were tested after the drying and LN exposure steps. With the encapsulation vitrification procedure, 5–10 tips each were recovered as controls after the preculture and encapsulation steps, while 10–15 tips each were recovered after the PVS2 and the LN exposure steps.

The percent of moisture remaining in samples of some beads that were air dried during the encapsulation dehydration procedure was determined gravimetrically by weighing the dried beads, placing them in an oven at 95°C overnight and then reweighing the beads. The percent moisture was calculated on a wet weight basis.

After LN exposure for at least 30 min, shoot tips from the encapsulation dehydration procedure were thawed at ambient temperature for 20 min and transferred to recovery plates of GM medium. Those from the encapsulation vitrification procedure were thawed in a 38°C water bath, rinsed with a solution of 1.2 M sucrose, and transferred to plates of GM medium. For recovery growth, shoot tips were incubated under the same temperature and light conditions used for culture maintenance. Survival was measured as the number of shoot tips remaining green and showing growth at 2 wk.

Data Analysis

For growth experiments, data for the 12 genotypes were combined to evaluate the effects of treatment, while data for treatments were combined to evaluate the effects of genotype. Data on shoot and root production and survival through cryopreservation were analyzed using StatView 5.0.1 (SAS Institute Inc.). One-way analysis of variance (ANOVA) was calculated and the significance of differences was determined by using the Tukey-Kremer post hoc test.

Genetic Analysis

Total genomic DNA was extracted from plants that were micropropagated. Micropropagated plants were initiated from material held at the Arboretum at Flagstaff, originally collected from the more northern of the two population clusters in the Sacramento Mountains. According to records, the collection sites were at or near the following areas of separated populations. HT4 was collected on a north-facing slope. HT3, 11 and 12 were collected about 2200 ft ESE of the collection site for HT4. Samples HT2, 5, 6, 7, 8, 9 and 10 occurred along a slope extending northeast to southwest about 6000 ft ESE of the collection site for HT4. HT1 was collected from a population about 7700 ft south of the collection site for HT4. All plants were collected at elevations above 6200 ft.

Weighed samples (ca. 100 mg) were frozen in LN, ground to a fine powder, and extracted by a modification of the CTAB procedure (Stewart and Via 1993). Yields of DNA of at least 30 µg were typical. Concentrations of DNA were determined using a Pharmacia Ultrospec Plus spectrophotometer (Uppsala, Sweden). The average ratio of absorbance at 260 nm/280 nm was 1.6. The apparent DNA concentration was adjusted to 50 µg/ml with TE buffer and used without further cleanup.

Polymerase chain reactions were run in an Idaho Technology (Salt Lake City, UT) Rapidcycler using borosilicate glass capillary tubes (part no. 1706). Samples were prepared and run in duplicate or triplicate. Each sample tube of 10 µl contained 50 mM Tris (pH 8), 4 mM $MgCl_2$, 20 mM KCl, 0.005 mg BSA, 0.1 mM dNTPs, 0.6 units TAQ (TaKaRa), 0.4 µM ten-base oligomer (ten-mer) and 10 ng genomic DNA. The amplification protocol was 1 min at 92°C followed by 2 cycles of 7 secs at 42°C, 70 secs at 72°C and 60 secs at 92°C, then 38 cycles of 7 secs at 42°C, 70 secs at 72°C, 1 sec at 92°C, with a final 4 min at 72°C. Ten-mers were obtained from Operon Technologies (now www.GeneLink.com) and their numbering system was used.

Gels were prepared by microwaving Seachem GTG agarose (BioWhittaker Molecular Applications, Rockland, Maine.) at 1.6% in TBA buffer (0.0005% ethidium bromide was added just before the 50°C agarose was poured). Samples from the capillary tubes (10 µl) were mixed with a 2 µl solution of 50% glycerol containing bromophenol blue and xylene cyanole as marker dyes. Comparison to a 100 bp ladder was used to estimate fragment size. Electrophoresis was carried out at 80 volts on a 5 × 8 cm gel. The separated fragments were visualized using a UV-transilluminator and photographed in black and white with a digital camera (Sony Mavica FD83). Photos were digitally enhanced to improve contrast using ArcSoft Photo Studio 2.0 (ArcSoft, Inc., Fremont, CA) software and printed on an HP Deskjet 890C. Scoring of RAPD bands was done directly on the printed output.

From an initial group of ten different ten-mers, four were selected based on the reproducible and discrete banding patterns in the RAPD assay: Operon numbers A11 (CAATCGCCGT), AB05 (CCCGAAGCGA), G17 (ACGACCGACA) and X03 (TGGCGCAGTG). The presence or absence of a total of 49 individual bands was scored as a character when a band was present in at least one of the twelve lines of micropropagated *H. todsenii*. Each band was considered a separate character, and information on the presence or absence of a band was analyzed using the Phylip package of programs (Nei's Distance Measure, RESTDIST, and the Neighbor-Joining program, NEIGHBOR). The resulting tree indicated the clustering of individual plants into groups based on number of bands in common (Felsenstein 1983, 1985; Backeljau et al. 1995).

Results

Shoot Propagation

An average of over 5 shoots per culture developed after two months on GM medium, but propagation rates were increased significantly on P medium (Table 2). Significantly less shoot propagation occurred on R medium, which was used to initiate rooting. There was no difference in the rate of shoot multiplication at 0, 0.5 or 2 mg/L IBA, but there was a small but significant increase in shoots on 10 mg/L IBA.

Propagation on GM was compared in stem pieces with and without apices. While there was propagation from both explants, there were significantly more shoots from apical pieces (6.37 ± 0.25) than from nodes of decapitated shoots (5.68 ± 0.25) ($P < 0.05$).

When shoot multiplication rates were compared between lines in these three experiments, there was only one consistent difference between lines. In each experiment, HT-9 was among the top three lines in the number of shoots produced, while HT-3 was always among the lowest three.

TABLE 2. AVERAGE NUMBER OF SHOOTS, ROOTS PRODUCED PER SHOOT, AND PERCENT OF SHOOTS ROOTING IN TWO EXPERIMENTS. 1) A comparison of three media commonly used in this laboratory; and 2) a comparison of four concentrations of IBA. All media contained MS basal medium with 0.8% agar. Lower case letters indicate statistical differences for the parameter measured within the experiment ($P < 0.01$).

Test	Medium	Shoots/culture	Roots/shoot	Rooting %
Experiment 1	GM	5.53 ± 0.16a	0.9 ± 0.11a	27.43 ± 6.5a
	P	8.10 ± 0.35b	1.33 ± 0.22a	29.85 ± 8.0a
	R	4.21 ± 0.14c	3.88 ± 0.26b	62.68 ± 8.5b
Experiment 2	0 IBA	2.80 ± 0.13a	2.82 ± 0.20ac	66.88 ± 3.59a
	0.5 IBA	3.25 ± 0.14a	3.61 ± 0.23a	69.95 ± 5.00a
	2 IBA	3.39 ± 0.17a	3.25 ± 0.23a	70.53 ± 4.06a
	10 IBA	4.23 ± 0.21b	2.09 ± 0.19c	50.65 ± 6.33a

There was also a significant difference between the number of shoots produced from these two lines in all three experiments ($P < 0.05$).

Rooting

When rooting was compared on GM, P, and R media, root initiation occurred on all three media, but there were significantly more shoots producing roots as well as significantly more roots per shoot on the IBA-containing R medium (Table 2). When higher levels of IBA were tested, they did not increase the rate of rooting, and a small decrease in the number of roots per shoot was seen on 10 mg/L compared with 0.5 and 2.0 mg/L IBA. Roots produced on medium with 10 mg/L IBA were generally longer than on other media, but they were more likely to be aerial and produced from nodes above the medium (data not shown). When genetic lines were compared in this experiment, lines HT-2, HT-4, and HT-5 had significantly more rooting than other lines ($P < 0.05$) in both experiments.

Rooting was also obtained from shoots that were cultured for 7 d on 0 or 50 mg/L IBA followed by two months on charcoal medium with no hormones. Rooting from the control (53.2 ± 7.5%) was not statistically different from the IBA treated shoots (65.3 ± 5.6%)

Acclimatization

Of 225 plants acclimatized in two rooting experiments, 54% were surviving at one month. There was no statistical difference in the survival rates of plants rooted with the IBA pulse and IBA concentration experiments and no differences in the survival of rooted plants from any of the individual treatments in those experiments or from different propagation media. There also appeared to be no effect of genetic line on survival through acclimatization in these trials.

Cryopreservation

Survival of shoot tips through cryopreservation ranged from 10–93%, with an average of 35%, but there was no significant difference in the average rates of survival using encapsulation dehydration (29.9 ± 4.5%) and encapsulation vitrification (37.4 ± 4.0%). Moisture levels were determined in five of the encapsulation dehydration experiments and ranged from 14.1% to 24.3%, with an average of 20.0%. In this range of moisture, survival ranged from 20% (24.3% moisture) to 53% (22.8% moisture) with no correlation between moisture and survival.

Analysis of survival data for the steps in the encapsulation dehydration procedure indicated that there was no significant difference in survival through the first three steps, but that survival decreased significantly with LN exposure ($P < 0.01$) (Fig. 1). Similarly, with the encapsulation vitrification procedure, there was a significant decrease in survival with LN exposure ($P < 0.01$).

Genetic Analysis

In spite of the small number of samples available from each location there was a remarkably consistent correlation of the within-population diversity with the collection location (Fig. 2). For example, HT1 and HT4 were collected from sites 1 and 4 that were separated by the greatest distance, and these had fewer characters in common. They also differed from HT3, HT11, and HT12, which were collected from several plants in the same locale but on a different mountain slope (site 2). HT2 and HT5-10 were collected from yet another slope (site 3) and, while they showed some differences, HT5, HT8, HT9 and HT10 were remarkably similar. These preliminary results suggest a possible association of low genetic diversity within populations and greater diversity between populations.

DISCUSSION

This work demonstrates that in vitro methods and biotechnology have the potential for playing a role in the conservation of *H. todsenii*. This species is found in three small isolated clusters of populations in the mountains of New Mexico and is threatened by its limited numbers, drought,

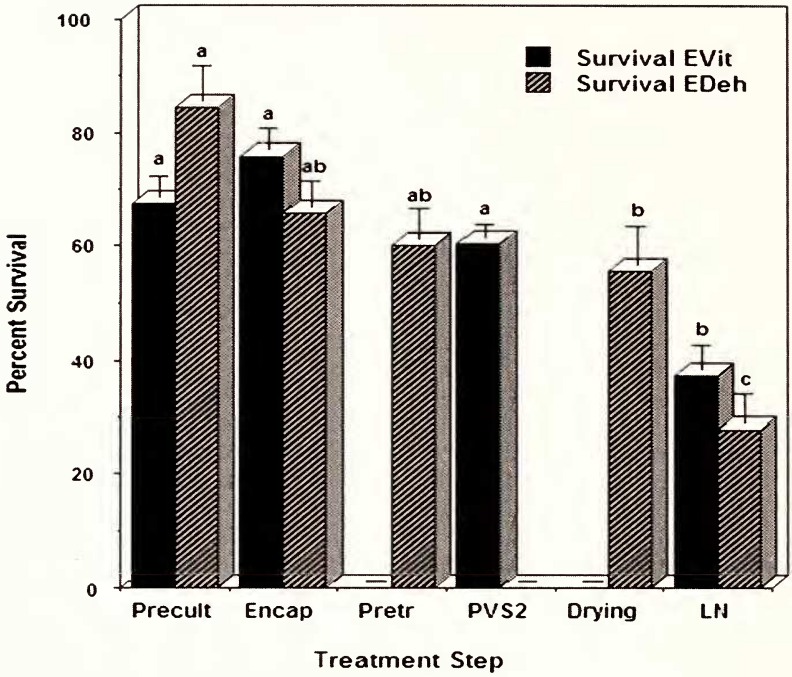

FIG. 1. Survival of shoot tips of *H. todsenii* through two cryopreservation protocols: encapsulation dehydration (EDeh) and encapsulation vitrification (EVit). Survival was tested after preculture (precult), encapsulation (encap), pretreatment (pretr) (EDeh only), PVS2 (EVit only), drying (EDeh only), and liquid nitrogen exposure (LN). For each protocol, different letters indicate significant differences ($P < 0.01$).

climate change, and its apparent limited ability for sexual reproduction. The methods described here can supplement traditional methods for propagation and germplasm analysis and storage, in order to provide a back-up for the natural population and to assist in its effective conservation.

The establishment of the in vitro cultures used in these experiments was done both by IVC and by processing cuttings sent to the lab by overnight shipment, demonstrating that either method can be utilized with this species. The media and methods used for propagating *H. todsenii* shoots in vitro are similar but not identical to those used elsewhere to propagate *H. multiflorum* (Koroch et al. 1997). With the latter, half-strength MS medium with a higher level of BAP was used for propagation, while indoleacetic acid (IAA) was used for rooting. With *H. todsenii*, higher concentrations of BAP resulted in hyperhydric shoots, and thus the low concentration of BAP in the GM medium was used. In the case of acclimatization of *H. todsenii*, the method used for propagation or rooting did not appear to affect survival *ex vitro*. Similarly, Koroch et al. (1997) observed no effect on survival of different sucrose concentrations used in preconditioning *H. multiflorum* for acclimatization.

This work was done as part of the Endangered Plant Propagation Program at CREW, where multiple endangered species are targeted for study at any one time. In dealing with a new species, a few media that have worked well with other species for propagation and rooting are tested first and then modified as needed. Since mass propagation on a commercial scale is not the goal, demonstrating the technique and producing a few hundred plants may be done without optimization of the media. In these studies with *H. todsenii*, three media that are used frequently in this lab were at the core of these studies, and both shoot and root initiation were measured on all of these. The protocols established have since been used to initiate cultures from shoots of new genotypes sent to CREW as shoots from both an ex situ collection and a wild population. Based on the results of these studies, the higher level of cytokinin (P medium) is used to initiate and build up the number of shoots in cultures, even though these cultures may tend to become hyperhydric if maintained at length on this medium. Then the lower hormone medium (GM) is used to maintain and normalize the cultures. Rooting is done on the R medium, as these studies indicated no benefit of increased IBA concentrations.

Shoot tips of *H. todsenii* survived cryopreservation protocols and were banked in liquid nitrogen for long-term storage. Successful cryopreservation of shoot tips has also been reported for other Lamiaceae species, including several

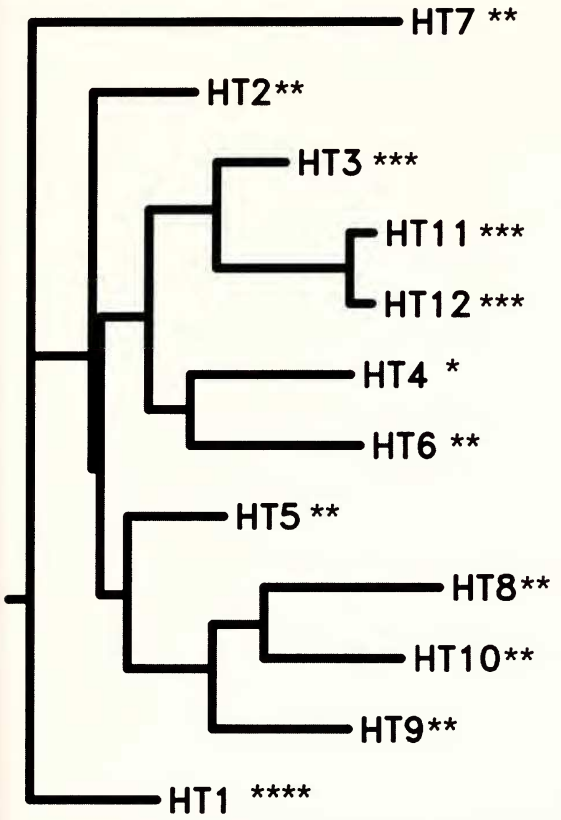

FIG. 2. A tree reflecting distance measures made from RAPD analyses of 12 lines of *H. todsenii*. The four collection sites are indicated by number of asterisks. Note the correlation of the similarity of RAPD characters with the geographical location of the plant.

species of *Mentha* and *Solemostemon rotundifolius* (Towill 1990; Hirai and Sakai 1999; Sakai et al. 2000; Niino et al. 2000) using encapsulation dehydration and vitrification procedures. The effects of cold-acclimation and of the vitrifying solution on *Mentha* shoot tips have also been studied (Volk et al. 2006; Senula et al. 2007). In a comparison of three cryopreservation methods using *Mentha* spp., controlled cooling gave the highest survival, but good survival was also observed using either encapsulation dehydration or vitrification, with some variation depending on the genotype (Uchendu and Reed 2008). In our studies, encapsulation dehydration and encapsulation vitrification gave comparable survival with *H. todsenii*.

The results of genetic analysis using RAPDs shows that intra-population diversity was less than that seen between populations, but inter-population diversity in this species was also low. However, only 12 lines were available for analysis, and these all came from only one of the three population clusters of this species: the northern cluster in the Sacramento Mountains. Future studies are planned to obtain more samples from the less accessible southern Sacramento cluster, as well as from the cluster in the San Andres Mountains, which is approximately 45 mi west of the two Sacramento Mountain clusters. Analysis of genotypes from these other areas will provide a more complete picture of the inter-population diversity of *H. todsenii*. It should also help direct ex situ conservation efforts by identifying areas with the most diversity within this species. Those areas could then be targets for more intense collecting and storage ex situ.

Obtaining additional genotypes will also provide further material for germplasm storage. Since seed production in this species is low and the populations are in remote areas, seeds are rarely available and seed banking is not an adequate option for long-term ex situ conservation. As a back-up to the maintenance of an actively growing ex situ collection at the Arboretum at Flagstaff, shoot tips from multiple genotypes of *H. todsenii* are being maintained at CREW in LN for long-term storage. Although more labor intensive than traditional seed banking, cryostorage of shoot tips may provide the only method currently available for ex situ banking of germplasm from species producing few or no seeds.

As an isolated mountain species, *H. todsenii* is among the species most vulnerable to the effects of climate change, while its small numbers make it vulnerable to drought, fire, or other catastrophic environmental events. In vitro methods provide a variety of tools that can aid in the conservation of the endangered *H. todsenii*. In vitro culture, which can be initiated by IVC, if needed, can readily propagate shoots that can be rooted in vitro, providing plants that can be acclimatized to soil. In vitro cultures provide shoot tips that can be cryopreserved as a substitute for seed banking. They also provide material that can be analyzed readily by RAPDs for genetic diversity, which, in turn, can provide direction for targeting further collections in areas of greatest diversity. These ex situ activities can, in turn, provide a back-up to conservation efforts to protect the species in situ and ensure the maintenance of *H. todsenii* into the future.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Dr. Joyce Maschinski and Sheila Murray at The Arboretum at Flagstaff for making the original collections and sending material from the ex situ collection at TAF for these studies, for providing information on the species, and for helpful comments; Dr. John R. Clark for preliminary studies, and Jennifer Rieger, Lisa Cleveland, Rachel Kennedy, Sean Carr, and Jodi Omnitz for technical assistance. The development of the propagation and cryopreservation protocols was supported, in part, by grants from the Institute of Museum and Library Services, received in collaboration with the Center for Plant Conservation (St. Louis, MO).

Literature Cited

Adams, R. P., A. E. Schwarzbach, and R. N. Pandey. 2003. The concordance of terpenoid, ISSR and RAPD markers, and ITS sequence data sets among genotypes: an example from *Juniperus*. Biochemical Systematics and Ecology 31:375–387.

Backeljau, T., L. De Bruyn, H. De Wop, K. Jordaem, S. Van Dongen, R. Verhagen, and B. Winnepenninckx. 1995. Random amplified polymorphic DNA (RAPD) parsimony methods. Cladistics 11:119–130.

Fabre, J. and J. Dereuddre. 1990. Encapsulation dehydration: a new approach to cryopreservation of *Solanum* shoot tips. CryoLetters 11:413–426.

Felsenstein, J. 1983. Parsimony in systematics: biological and statistical issues. Annual Review of Ecology and Systematics 14:313–333.

———. 1985. Confidence limits in phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Hirai, D., K. Shirai, S. Shirai, and A. Sakai. 1998. Cryopreservation of *in vitro*-grown meristems of strawberry (*Fragaria x ananassa* Duch.) by encapsulation-vitrification. Euphytica 101:109–115.

——— and A. Sakai. 1999. Cryopreservation of *in vitro*-grown axillary shoot-tip meristems of mint (*Mentha spicata* L.) by encapsulation vitrification. Plant Cell Reports 19:150–155.

Huenneke, L. F. 1993. Interaction of breeding system and genetic structure in *Hedeoma todsenii* (Lamiaceae), a rare mint of New Mexico, Final report to Center for Plant Conservation and New Mexico Forestry and Resources Conservation Division, Santa Fe, NM.

Irving, R. S. 1979. *Hedeoma todsenii* (Labiatae), a new and rare species from New Mexico. Madroño 26:184–187.

Khanuja, S. P. S., A. K. Shasany, A. Srivastava, and S. Kumar. 2000. Assessment of genetic relationships in *Mentha* species. Euphytica 111: 121–125.

Koroch, A. R., H. R. Juliani, Jr., H. R. Juliani, and V. S. Trippi. 1997. Micropropagation and acclimatization of *Hedeoma multiflorum*. Plant Cell Tissue and Organ Culture 48:213–217.

Krasnyanski, S., T. M. Ball, and K. C. Sink. 1998. Somatic hybridization in mint: identification and characterization of *Mentha piperita* (x) *M. spicata* hybrid plants. Theoretical and Applied Genetics 96:683–687.

Linsmaier, E. M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum 18:100–127.

Liu, J., L. Wang, Y. Geng, Q. Wang, L. Luo, and Y. Zhong. 2006. Genetic diversity and population structure of *Lamiophlomis rotata* (Lamiaceae), an endemic species of Qinghai–Tibet Plateau. Genetica 128:385–394.

Lloyd, G. and B. McCown. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip cultures. Proceedings of the International Plant Propagators' Society 30:421–427.

Maki, M. and S. Horie. 1999. Random amplified polymorphic DNA (RAPD) markers reveal less genetic variation in the endangered plant *Cerastium fischerianum* var. *molle* than in the widespread conspecific *C. fischerianum* var. *fischerianum* (Caryophyllaceae). Molecular Ecology 8:145–150.

Mattner, J., G. Zawko, M. Rossetto, S. L. Krauss, K. W. Dixon, and K. Sivasithamparam. 2002. Conservation genetics and implications for restoration of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from Western Australia. Biological Conservation 107:37–45.

Morden, C. W. and W. Loeffler. 1999. Fragmentation and genetic differentiation among subpopulations of the endangered Hawaiian mint *Haplostachys haplostachya* (Lamiaceae). Molecular Ecology 8:617–625.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473–497.

Niino, T., A. Hettiarachchi, J. Takasashi, and P. K. Samarajeewa. 2000. Cryopreservation of lateral buds of in vitro-grown innala plants (*Solemostemon rotundifolius*) by vitrification. CryoLetters 21:349–356.

Pence, V. C. 2005. In vitro collecting (IVC) I. The effect of media and collection method on contamination in temperate and tropical collections. In vitro Cellular and Developmental Biology-Plant 41:324–332.

———, J. A. Sandoval, V. M. Villalobos A., and F. Engelmann (eds.). 2002. In vitro collecting techniques for germplasm conservation. IPGRI Technical Bulletin 7. International Plant Genetic Resources Institute, Rome, Italy.

Sakai, A., T. Matsumoto, D. Hirai, and T. Niino. 2000. Newly developed encapsulation-dehydration protocol for plant cryopreservation. CryoLetters 21:53–62.

Senula, A., E. R. J. Keller, and T. Sanduijav. 2007. Cryopreservation of cold-acclimated mint (*Mentha* spp.) shoot tips using a simple vitrification protocol. CryoLetters 28:1–12.

Skoula, M., I. El Hilali, and A. M. Makris. 1999. Evaluation of the genetic diversity of *Salvia fruticosa* Mill. clones using RAPD markers and comparison with the essential oil profiles. Biochemical Systematics and Ecology 27:559–568.

Stewart, C. N., Jr. and L. E. Via. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques 14:748–751.

Sydes, M. and R. Peakall. 1998. Extensive clonality in the endangered shrub *Haloragodendron lucasii* (Haloragaceae) revealed by allozymes and RAPDs. Molecular Ecology 7:87–93.

Towill, L. E. 1990. Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Reports 9:178–180.

Uchendu, E. E. and B. M. Reed. 2008. A comparative study of three cryopreservation protocols for effective storage of *in vitro*-grown mint (*Mentha* spp). CryoLetters 29:181–188.

U.S. Fish and Wildlife Service. 2001, Todsen's pennyroyal (*Hedeoma todsenii*) revised recovery plan. U.S. Fish and Wildlife Service, Albuquerque, NM.

Volk, G. M., J. L. Harris, and K. E. Rotindo. 2006. Survival of mint shoot tips after exposure to cryoprotectant solution components. Cryobiology 52:305–308.